REMARKS

Claims 1-16 and 18-21 are pending in the application.

I. Claim Rejections—35 U.S.C. §102(b) over Capecchi

Claims 1, 4, 6, 8, 10, 12 and 18-20 stand rejected under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 5,464,764 issued to Capecchi et al. (hereinafter "Capecchi").

Although Capecchi teaches vectors comprising a positive and a negative selection marker and homologous regions, the vectors of Capecchi are significantly different from the vector of Applicant's claimed construct in at least two aspects. First, Capecchi's vectors do not contain two direct repeat sequences. Claim 1 of the instant application requires that the construct comprises "two direct repeats of a gene of interest, each direct repeat comprising a nucleic acid sequence encoding a peptide, wherein the peptide is capable of being expressed in said plant cells...."

The claimed construct of the instant application contains a positive marker and a negative marker, which are flanked by two direct repeats. See Paragraphs 5-7 on pages 2-3 of the original Specification. Paragraphs 5-7 on pages 2-3 also teaches introduction of this construct into a plant cell, and selecting for plant transformant using the positive selectable marker. Figures 1A and 1B of the Specification show that the direct repeats immediately flank the two marker genes and that each direct repeat comprises a nucleic acid sequence encoding a peptide, wherein the peptide is capable of being expressed in said plant cells. Paragraph 35 on page 10 further shows the presence of two copies of the gene of interest flanking the marker genes. Thus, the instant claims require that the two direct repeats each contains a copy of a gene of interest.

By contrast, Capecchi does not specify that the two homologous regions of the vector are direct repeats of the same gene. More specifically, Capecchi teaches that the "PNS vector comprises four DNA sequences. The first and second DNA sequences each contain portions which are substantially homologous to corresponding homologous portions in first and second regions of the targeted DNA. Substantial homology is necessary between these portions in the PNS vector and the target DNA to insure targeting of the PNS vector to the appropriate region of the genome." Col. 6, lines 51-58 of Capecchi. There is no teaching in Capecchi that the first and

second regions of the targeted genomic DNA are the same, it is thus reasonable to conclude that the first and second DNA sequences on the vector are not the same because they are homologous to each other, respectively. Thus, it is more likely than not that the first and second regions of Capecchi are not direct repeats of a gene of interest.

Secondly, the instant claims recite a construct comprising a positive and a negative selectable marker, and two direct repeats immediately flanking the positive and a negative selectable marker gene. Although Capecchi also discloses a positive and a negative selectable marker, Capecchi never shows that the two homologous direct repeats immediately flank the positive and negative selectable marker genes. The Examiner stated that Fig. 1 depicts a vector with just a positive marker. Applicant respectfully disagrees. Fig. 1 of Capecchi clearly shows that the first and second homologous regions flanking the positive selectable marker (3rd DNA sequence), with the negative selectable marker (4th DNA sequence) residing outside of the segment flanked by the first and second homologous regions.

Thus, because not every limitation of the instant claims are taught or suggested by the cited reference, withdrawal of the section 102 rejection is respectfully requested.

II. Claim Rejections—35 U.S.C. §103(a) over Peterson, in view of Bauer and Lassner

Claims 1-7, 10-16, 18 and 21 stand rejected under 35 U.S.C. §103(a) as being obvious over U.S. Patent No. 6,984,774 issued to Peterson (hereinafter "Peterson"), in view of U.S. Patent No. 6,534,315 issued to Bauer (hereinafter "Bauer"), and further in view of U.S. Patent Publication No. 2002/0035739 by Lassner (hereinafter "Lassner"). Applicants respectfully disagree for reasons set forth below.

Obviousness is a question of law based on underlying factual inquiries. The factual inquiries enunciated by the U.S. Supreme Court in *KSR Int'l C. v. Teleflex, Inc.*, 127 S. Ct. 1727, 82 USPQ2d 1385 (2007) include the *Graham* factors of determining the scope and content of the prior art, ascertaining the differences between the claimed invention and the prior art, and resolving the level of ordinary skill in the pertinent art.

Once the *Graham* factual inquiries are resolved, the Examiner must explain why the difference(s) between the cited references and the claimed invention would have been obvious to one of ordinary skill in the art. The rationale used must be a permissible rationale. The USPTO

has promulgated examination guidelines for determining obviousness in view of *KSR* in M.P.E.P. §2143(A)-(G). These *KSR* Guidelines enumerate permissible rationales and the findings of fact that must be made under the particular rationale.

(i) The teaching of the cited references

Peterson relates to "methods and materials to induce homologous recombination in a plant, comprising introducing a recombination construct to a plant and making available to the plant a transposase, so as to induce recombination." *See* abstract of Peterson. Peterson discloses a construct containing a Ds element flanked by direct repeats or overlapping sequences. Col. 3, lines 25-42 of Peterson. Peterson further teaches that the construct may optionally contain a selectable marker. Col. 3, lines 43-44 of Peterson. The only example provided by Peterson is a construct with a Ds element located between two partially overlapping non-functional segments of the GUS gene. Col. 9, lines 35-39; *see also* Fig. 1 of Peterson.

Bauer does not teach direct repeats that can lead to expression of the peptide encoded by the direct repeat. On the contrary, Bauer teaches how the direct repeats should be rendered noncoding. *See e.g.*, lines 45-48, Col. 4 and lines 22-28, Col. 7 of Bauer, stating "... made noncoding by any appropriate means such as changing the reading frame or the introduction of stop codons."

Lassner relates to methods for identifying plant disease resistant genes and is only relied upon by the Examiner to show that a negative selectable marker can be used in plant engineering.

(ii) Substantial differences exist between the cited references and Applicant's invention.

Applicant respectfully submits that substantial differences exist between the three cited references and the present invention for the following reasons:

First, although Peterson teaches that the construct may contain two direct repeats, Peterson never specifically teaches or suggests that each of the two repeats comprises a nucleic acid sequence encoding a peptide that is capable of being expressed in the plant. Indeed, Peterson states that the two direct repeats can encode a gene product when recombined, but never mentions that each direct repeat alone comprises a nucleic acid sequence encoding a peptide that is capable of being expressed in the plant, as is taught and claimed by Applicant.

The Examiner maintains that the direct repeat of Peterson necessarily encode at least a dipeptide, Applicant respectfully disagrees. Because Peterson only contemplates that the two direct repeats can encode a gene product when recombined, it is purely speculative to argue that the two direct repeats of Peterson can each encode a peptide before they are recombined. It is well known that any given DNA sequence may be a coding sequence or a noncoding sequence. A coding DNA sequence may be rendered noncoding by, for example, the introduction of a frameshift or a Stop codon. It is also well known that translation of protein from a mRNA is a regulated process. Not all random mRNA containing a coding sequence capable of encoding a dipeptide will eventually be expressed in an eukaryotic organism, such as a plant. For instance, as the research paper by Zheng et al. (Appendix 1) shows, mRNAs containing upstream nonsense (Stop) codon may be specifically targeted for degradation before they can be translated into a peptide. Thus, the Examiner's position that each direct repeat of Peterson necessarily contains a coding sequence capable of being expressed in the plant can not stand.

Applicant's position is also supported by Bauer which teaches that the direct repeat sequences (DRS) can be "made noncoding by any appropriate means such as changing the reading frame or the introduction of stop codons." See Col. 7, lines 25-28 of Bauer. Even if Stop codons are introduced into the DRS of about 200 bp long, there likely to be two contiguous codons that are not Stop codons. Thus, if the Examiner were correct, Bauer would have to change every other codons into a Stop codon in order to made the DRS noncoding. That is not what one of skills in the art understands the terms "coding" and noncoding" to mean. Taken together, each of the direct repeats of Peterson does not inherently encode a peptide that is capable of being expressed in the plant.

Secondly, even if we assume that the direct repeats of Peterson were the same as those of the instant invention, the direct repeats of Peterson do not immediately flank the positive and negative markers, which is required by Applicant's claims 1, 4 and 21. According to the specification and Fig. 1 of Peterson, the Ds element is located between the positive marker gene and the direct repeat. Therefore, the marker gene of Peterson is not immediately flanked by the direct repeats on both sides, as is required by Applicant's claims. The Ds element is required for operation of the invention disclosed in Peterson and cant not be readily removed without rendering the Peterson invention inoperable. *See e.g.*, Col. 2, lines 46-49 of Peterson, stating that

the "invention is directed to the unexpected finding that overlapping foreign gene sequences containing a maize Ds element can be induced to undergo homologous recombination upon introduction of the maize Ac transposase."

The Examiner maintains that Bauer teaches two direct repeats immediately flanking a positive and negative markers and thus can cure the defect of Peterson. See page 11 of the Office Action dated 3/30/09. Applicant respectfully disagrees. First, the direct repeats of Bauer are different in kind from the direct repeats of Peterson in that the direct repeats of Bauer are noncoding and nonrecombinogenic. See Col. 4, lines 4-7 of Bauer. Secondly, while the direct repeats of the instant claims immediately flank the selectable markers, the direct repeats of Bauer are separated by other sequences, such as the E. coli replication origin or a yeast replication origin. See e.g., Col. 5, lines 5-21 of Bauer. Thus, significant difference exist between the instant claims and the disclosure of the cited references.

(iii) It would not have been obvious to modify the teaching of the cited references to arrive at Applicant's invention.

Not only does the teaching of the cited references substantially different from Applicant's invention, but the differences are not such that it would have been obvious for one of ordinary skill in the art to modify the teaching of the cited references to arrive at Applicant's invention.

First, Bauer is not to be combined with Peterson because doing so would render the prior art unsatisfactory for its intended purpose or change the principle of operation of at least one of the references. In discussing obviousness rejection by combining multiple references, the MPEP states that a "proposed modification cannot render the prior art unsatisfactory for its intended purpose or change the principle of operation of a reference." MPEP 2145. The Examiner relies on Bauer to teach a construct with a positive and negative selectable markers flanked by two direct repeats, one on each side. The Examiner further relies on Peterson to disclose two direct repeats that encode a peptide capable of being expressed in the plant. In doing so, the Examiner ignores the fact that Bauer cannot be combined with Peterson because Bauer specifically teaches that the direct repeat sequence is noncoding, i.e., not to be translated into a peptide. To combine Bauer with Peterson would render Bauer unsatisfactory for its intended purpose because one of the objectives of Bauer is to leave behind NO direct repeat sequence that encodes a peptide after excision. *See e.g.*, Col. 4, lines 35-48 of Bauer.

Secondly, even if the teachings of Peterson, Bauer and Lassner are combined, Applicant's claimed invention is not obvious because the Examiner has not provided any rationale as to why one of ordinary skill would be motivated to modify the direct repeats of Peterson to obtain Applicant's claimed construct. As explained above, Peterson never mentions that each direct repeat encodes a peptide. Rather, Peterson teaches that the direct repeats encode a peptide after recombination. This is in contrast to Applicant's invention where each direct repeat comprises a nucleic acid sequence that as so that the modified repeats would each encode a peptide without requiring recombination. The Examiner has not established why one of skill in the art would have found it obvious to modify the direct repeat of Peterson in order to arrive at Applicant's construct.

Last but not the least, the Examiner has not provided any motivation either in the references or in the common knowledge of one of ordinary skill in the art to remove the Ds element in Peterson's construct. As explained above, the Ds element is sitting between the selectable marker and one of the direct repeats. In order to arrive at Applicant's claimed invention, the Ds element need to be removed so that the selectable markers are immediately flanked by two direct repeats with each direct repeat encoding a peptide capable of being expressed in the plant cells. However, removing the Ds element would have rendered the Peterson invention inoperable.

Applicant recognizes that the references need to be considered as a whole, however, it is the conflicting teachings of the references that would discourage one of ordinary skill in the art to combine their teachings. Even if one is to combine these references, one would not find it obvious to reconcile these conflicting teachings in order to arrive at Applicant's claimed invention. As the Supreme Court warned against in Graham and reiterated in KSR, a fact finder must resist the temptation to read into the prior art the teachings of the invention at issue. Applicant respectfully submits that the Examiner has not established that one of ordinary skill in the art would be motivated to modify the conflicting teachings of the three cited references in a manner claimed by Applicant without slipping into the use of hindsight.

Thus, because substantial differences exist between the cited references and Applicant's claimed invention, and because such differences would not have been obvious to one of skill in

the art at the time of Applicant's invention, Applicant's invention is not rendered obvious by the cited references.

III. Claim Rejections—35 U.S.C. §103(a) over Peterson, in view of Bauer, Lassner, and Capecchi

Claims 1-16, and 18-21 stand rejected under 35 U.S.C. §103(a) as being obvious over Peterson, in view of Bauer and Lassner, and further in view of Capecchi. Applicant respectfully disagrees for reasons set forth below.

For reasons set forth above in Section II, Peterson, Bauer and Lassner do not render the presently claimed invention obvious. As explained in Section I, Capecchi does not cure the defect because it fails to teach that the direct repeat sequences immediately flank the positive and negative selectable markers. Thus, because substantial differences exist between the 4 cited references and Applicant's claimed invention, and because such differences would not have been obvious to one of skill in the art at the time of Applicant's invention, Applicant's invention is not rendered obvious by the cited references.

Based upon the foregoing discussion, Applicants' attorney submits that the amended claims are in a form for allowance and respectfully solicits a Notice of Allowance. The Commissioner is authorized to charge the required fee for a two month extension of time to deposit account 12-0600.

Respectfully submitted,

LATHROP & GAGE LLP
Attorneys for Applicants:

Xiaoyue Chen, Reg. No. 51,552
4845 Pearl East Circle, Suite 201
Boulder, CO 80301
Phone: (720) 931-3014
Fax: (720) 931-3001

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Nonsense-Mediated Decay of *ash1* Nonsense Transcripts in *Saccharomyces cerevisiae*

Wei Zheng,* Jonathan S. Finkel,* Sharon M. Landers,† Roy M. Long† and Michael R. Culbertson*,[1]

**Laboratories of Genetics and Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706 and* †*Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226*

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ABSTRACT

Nonsense-mediated mRNA decay (NMD) performs two functions in eukaryotes, one in controlling the expression level of a substantial subset of genes and the other in RNA surveillance. In the vast majority of genes, nonsense mutations render the corresponding transcripts prone to surveillance and subject to rapid degradation by NMD. To examine whether some classes of nonsense transcripts escape surveillance, we asked whether NMD acts on mRNAs that undergo subcellular localization prior to translation. In *Saccharomyces cerevisiae*, wild-type *ASH1* mRNA is one of several dozen transcripts that are exported from the mother-cell nucleus during mitotic anaphase, transported to the bud tip on actin cables, anchored at the bud tip, and translated. Although repressed during transport, translation is a prerequisite for NMD. We found that *ash1* nonsense mutations affect transport and/or anchoring independently of NMD. The nonsense transcripts respond to NMD in a manner dependent on the position of the mutation. Maximal sensitivity to NMD occurs when transport and translational repression are simultaneously impaired. Overall, our results suggest a model in which *ash1* mRNAs are insensitive to NMD while translation is repressed during transport but become sensitive once repression is relieved.

IN eukaryotes, nonsense-mediated mRNA decay (NMD) plays a role in RNA surveillance by eliminating aberrant transcripts that contain a nonsense or frameshift mutation, thereby preventing the accumulation of potentially deleterious dominant-negative proteins. In addition, a subset of functional, error-free mRNAs accumulate in a manner dependent on the NMD pathway in the yeast *Saccharomyces cerevisiae* (Guan *et al.* 2006), including transcripts with a small upstream open reading frame that initiates translation in the 5′-untranslated region (UTR) (Oliveira and McCarthy 1995), transcripts in which an internal out-of-frame open reading frame (ORF) is translated due to inefficient translation initiation at the first AUG codon (Welch and Jacobson 1999), and precursors that undergo inefficient splicing in which the intron contains an in-frame stop codon (He *et al.* 1993).

The *UPF1*, *UPF2*, and *UPF3* genes are required for NMD in *S. cerevisiae* (Leeds *et al.* 1992). The similarities of *UPF* gene orthologs from different classes of organisms coincide with similarities in the pathways for NMD, including a recruitment step initiated in the nucleus involving the nucleo-cytoplasmic shuttling protein Upf3p (Shirley *et al.* 1998, 2002; Serin *et al.* 2001), followed by translation initiation, premature termination, decapping, and decay in the cytoplasm. Although NMD can trigger RNA decay during any round of translation in yeast (Maderazo *et al.* 2003), decay is known to occur during the pioneer round of translation while RNAs are still bound to the nuclear cap-binding complex (Gao *et al.* 2005).

During pioneer translation, NMD appears to be temporally and spatially coupled to nuclear export. However, in *S. cerevisiae*, >25 transcripts have been identified where nuclear export and translation are separated by an intervening step in which the transcripts localize via translocation on actin cables. During transport, translation is repressed. Upon arrival and anchoring at the bud tip, translational repression is relieved (Long *et al.* 1997; Takizawa *et al.* 2000; Shepard *et al.* 2003; Andoh *et al.* 2006; Aronov *et al.* 2007). *ASH1* translation appears to utilize specialized ribosomes containing a specific subset of paralogous ribosomal proteins (Komili *et al.* 2007; Warner 2007). These exceptional transcripts can be exploited to learn more about NMD.

ASH1 mRNA, the best-studied transcript that localizes via actin cables, codes for a transcriptional repressor of the *HO* gene, which produces the endonuclease that initiates homothallic switching between a- and α-mating types (Kruse *et al.* 2002; Gonsalvez *et al.* 2005; Zarnack and Feldbrügge 2007). Asymmetric localization of the

[1]*Corresponding author:* Laboratory of Molecular Biology, 1525 Linden Dr., University of Wisconsin, Madison, WI 53706.
E-mail: mrculber@wisc.edu

ASH1 transcript prior to translation leads to asymmetric competence to switch mating type (Chartrand *et al.* 2002). *ASH1* is transcribed in the mother-cell nucleus during anaphase (Long *et al.* 1997; Takizawa *et al.* 1997). She2p is hypothesized to bind *ASH1* mRNA in the nucleus (Kruse *et al.* 2002). Once in the cytoplasm, the She2p-*ASH1* ribonucleoprotein particle associates with Myo4p (She1p), a type V myosin motor protein, through the adaptor protein She3p (Gonsalvez *et al.* 2005). As a result of these associations, *ASH1* mRNA is tethered to a polarized actin cytoskeleton (Long *et al.* 1997; Takizawa *et al.* 1997).

During transport, translation of *ASH1* mRNA is slowed by She2p bound at three locations in the ORF and by two translational repressors, Khd1p and Puf6p, which bind the mRNA in the 5′- and 3′-UTR, respectively (Chartrand *et al.* 2002; Gu *et al.* 2004; Paquin *et al.* 2007; Deng *et al.* 2008). Another protein, Loc1p, which affects 60S rRNA processing and ribosome assembly (Harnpicharnchai *et al.* 2001; Urbinati *et al.* 2006), represses translation and is required for anchoring at the bud tip (Long *et al.* 2001). Upon arrival at the bud tip, *ASH1* mRNA is hypothesized to be anchored and translational repression is relieved (Gonzalez *et al.* 1999; Gu *et al.* 2004; Paquin *et al.* 2007; Deng *et al.* 2008). The *ASH1* transcript cofractionates with membranes, suggesting the possibility that it may be translated by membrane-associated ribosomes (Diehn *et al.* 2000). Locally produced Ash1p is subsequently imported into the daughter-cell nucleus to repress transcription of *HO*.

None of the localized mRNAs, including *ASH1*, are natural targets of NMD (Diehn *et al.* 2000), raising the possibility that localized mRNAs that contain a nonsense mutation might be also be immune to RNA surveillance. Support for this idea came from a report that a nonsense mutation at the 5′-end of the *ASH1*-coding region had no effect on mRNA abundance (Gonzalez *et al.* 1999). To further test whether or not representative asymmetrically localized transcripts are prone to RNA surveillance through NMD, we examined the behavior of *ash1* nonsense mRNAs containing mutations that terminate translation prematurely at three positions in the coding region. The results show that premature termination of translation affects mRNA localization independently of NMD. The degree of sensitivity of *ash1* nonsense transcripts to NMD is influenced by the position of the nonsense mutation, the transport system, and proteins that mediate translational repression. Our results are consistent with a model presented in the discussion that is based on the postulated existence of two subpopulations of transcripts: a translationally repressed, NMD-insensitive pool and a translatable, NMD-sensitive pool. The two-pool model explains many of the phenotypes of *ash1* nonsense mutations that are atypical with respect to NMD.

MATERIALS AND METHODS

Strains, plasmids, and genetic methods: Strains and plasmids used are listed in Tables 1 and 2, respectively. Yeast transformation was performed by electroporation (Grey and Brendell 1992) or the LiAc method (Gietz and Woods 2002) Growth media were described previously (Gaber and Culbertson 1982). Yeast gene deletions were constructed using the PCR-based gene disruption method (Baudin *et al.* 1993; Wach *et al.* 1994). The accumulation and decay of nonsense and missense mRNAs were analyzed in congenic strains expressing the genes from *CEN* plasmids and/or chromosomally integrated alleles constructed by gene replacement.

Allele construction: Full-length *ASH1* was PCR cloned into the centromeric (*CEN*) vector pRS314, including 500 nucleotides 5′ of the *ASH1* ORF and all sequences between the *ASH1* stop codon and the start codon of the next downstream gene, *SPE1*. Site-directed PCR mutagenesis was performed to generate nonsense and missense mutations. Base substitutions were introduced at three sites in the 1750-nucleotide *ASH1* ORF: +308 (site A), +968 (site B), and +1511 (site C) (Figure 1). Sites were chosen to meet three criteria:

A. Sequences in zip code regions E1, E2A, and E2B were avoided. These regions are binding sites for She2p and are required for localization (Chartrand *et al.* 1999; Gonzalez *et al.* 1999).
B. At least one consensus downstream element (TGYYGAT GYYYYY) thought to be required for NMD (Ruiz-Echevarria and Peltz 1996) was located within 200 nucleotides 3′ of each mutation.
C. The nonsense codons created by base substitution were followed by an A residue, which results in optimal translation termination and efficiency of NMD (Bonetti *et al.* 1995).

Mutant alleles of *ASH1* were chromosomally integrated using two-step gene replacement (Orr-Weaver and Szostak 1983). The integrity of integrated alleles was confirmed by DNA sequence analysis. To construct congenic strains, the following genes were disrupted by one-step gene replacement (Rothstein 1991): *UPF1*, *UPF2*, *UPF3*, *SHE2*, *SHE3*, *SHE4*, *SHE5*, *KHD1*, *PUF6*, or *LOC1*. Strains carrying *ASH1* alleles in a *she1*Δ background were identified among progeny from genetic crosses.

RNA methods: RNA isolation and Northern blotting were described previously (Shirley *et al.* 1998). Rates of RNA decay were determined by temperature shift of *rpb1-1* strains from 28° to 39° or by transcription inhibition using 10 μg/ml thiolutin (Parker *et al.* 1991). Cells harvested before temperature shift or drug addition (t_0) and at subsequent intervals were frozen in dry ice/ethanol. Total RNA was extracted and relative mRNA abundance was determined by quantitative RT–PCR using 18s rRNA as a loading control or by Northern blotting using *SCR1* mRNA as loading control. Half-lives were based on average values from three trials. SigmaPlot was used to evaluate decay data using the mixed exponential decay formula $y = a \times \exp(-b \times x) + c \times \exp(-d \times x)$ or the simple exponential decay formula $y = a \times \exp(-b \times x)$. Estimations of b, designated as B, and corresponding standard errors, designated as SE(B), were used to calculate standard error ($t_{1/2} = \log(2)/B$). $t_{1/2} \pm \mathrm{SE}(t_{1/2})$ was calculated as $[\log(2)/(B + \mathrm{SE}(B)), \log(2)/(B - \mathrm{SE}(B))]$.

Immunoprecipitation: Immunoprecipitation (IP) of She2p-cmyc or HA-Upf1p was performed as in Irie *et al.* (2002) with modifications. Exponentially growing yeast cultures (50 ml) were harvested at $OD_{600} = 0.6$. Cells were disrupted with acid-washed glass beads in 500 μl of lysis buffer containing

TABLE 1

Strains

Strain	Relevant genotype
W303a-ash1Δ	*ash1Δ::KanMX4 leu2-3,112 his3-11,15 ura3-1 trp1-1*
AAY320-ash1Δ	*ash1Δ::KanMX4 upf1Δ::URA3 leu2-3,112 his3⁻ ura3-1 trp1-1*
AAY334-ash1Δ	*ash1Δ::KanMX4 leu2-3,112 his3⁻ ura3⁻ trp1-1 rpb1-1*
AAY335-ash1Δ	*ash1Δ::KanMX4 upf1Δ::URA3 leu2-3,112 his3⁻ ura3⁻ trp1-1 rpb1-1*
K4452-she2Δ	*she2Δ::URA3 HO-CAN1 ade2-1*
K4452she2Δash1Δ	*ash1Δ::KanMX4 she2Δ::URA3 HO-CAN1 ade2-1*
ZWY3	*leu2-Δ0 met15-Δ0 his3-Δ1 ura3-Δ0*
ZWY3-khd1Δ	*khd1Δ::KanMX4*
ZWY3-loc1Δ	*loc1Δ::KanMX4*
ZWY3-puf6Δ	*puf6Δ::KanMX4*
ZWY3-she1Δ	*she1Δ::KanMX4*
ZWY3-she3Δ	*she3Δ::KanMX4*
ZWY3-she4Δ	*she4Δ::KanMX4*
ZWY3-she5Δ	*she5Δ::KanMX4*
ZWY7	*ash1-A-ns1 leu2-Δ0 met15-Δ0 his3-Δ1 ura3-Δ0*
ZWY7-khd1Δ	*ash1-A-ns1 khd1Δ::KanMX4*
ZWY7-loc1Δ	*ash1-A-ns1 loc1Δ::KanMX4*
ZWY7-puf6Δ	*ash1-A-ns1 puf6Δ::KanMX4*
ZWY7-she1Δ	*ash1-A-ns1 she1Δ::KanMX4*
ZWY7-she3Δ	*ash1-A-ns1 she3Δ::KanMX4*
ZWY7-she4Δ	*ash1-A-ns1 she4Δ::KanMX4*
ZWY7-she5Δ	*ash1-A-ns1 she5Δ::KanMX4*
ZWY14	*upf1Δ::LEU2*
ZWY14-khd1	*khd1Δ::KanMX4 upf1Δ::LEU2*
ZWY14-loc1Δ	*loc1Δ::KanMX4 upf1Δ::LEU2*
ZWY14-puf6Δ	*puf6Δ::KanMX4 upf1Δ::LEU2*
ZWY14-she1Δ	*she1Δ::KanMX4 upf1Δ::LEU2*
ZWY14-she3Δ	*she3Δ::KanMX4 upf1Δ::LEU2*
ZWY14-she4Δ	*she4Δ::KanMX4 upf1Δ::LEU2*
ZWY14-she5Δ	*she5Δ::KanMX4 upf1Δ::LEU2*
ZWY15	*ash1A-ns1 upf1Δ::LEU2*
ZWY15-khd1Δ	*ash1-A-ns1 khd1Δ::KanMX4 upf1Δ::LEU2*
ZWY15-loc1Δ	*ash1-A-ns1loc1Δ::KanMX4 upf1Δ::LEU2*
ZWY15-puf6Δ	*ash1-A-ns1puf6Δ::KanMX4 upf1Δ::LEU2*
ZWY15-she1Δ	*ash1-A-ns1 she1Δ::KanMX4 upf1Δ::LEU2*
ZWY15-she3Δ	*ash1-A-ns1 she3Δ::KanMX4 upf1Δ::LEU2*
ZWY15-she4Δ	*ash1-A-ns1 she4Δ::KanMX4 upf1Δ::LEU2*
ZWY15-she5Δ	*ash1-A-ns1 she5Δ::KanMX4 upf1Δ::LEU2*
ZWY8	*ash1-B-ns1 leu2-Δ0 met15-Δ0 his3-Δ1 ura3-Δ0*
ZWY11	*aAsh1-C-ns leu2-Δ0 met15-Δ0 his3-Δ1 ura3-Δ0*
ZWY16	*ash1-B-ns1 upf1Δ::LEU2*
ZWY17	*ash1-C-ns upf1Δ::LEU2*
ZWY21	*she2Δ::KanMX4*
ZWY22	*ash1-A-ns1 she2Δ::KanMX4*
ZWY23	*ash1-B-ns1 she2Δ::KanMX4*
ZWY24	*ash1-C-ns she2Δ::KanMX4*
ZWY25	*she2Δ::KanMX4 upf1Δ::LEU2*
ZWY26	*ash1-A-ns1 she2Δ::KanMX4 upf1Δ::LEU2*
ZWY27	*ash1-B-ns1 she2Δ::KanMX4 upf1Δ::LEU2*
ZWY28	*ash1-C-ns she2Δ::KanMX4 upf1Δ::LEU2*
ZWY29	*upf3Δ::URA3*
ZWY30	*ash1-A-ns1 upf3Δ::URA3*
ZWY31	*ash1-B-ns1 upf3Δ::URA3*
ZWY32	*ash1-C-ns upf3Δ::URA3*
ZWY37	*upf2Δ::URA3*
ZWY38	*ash1-A-ns1 upf2Δ::URA3*
ZWY39	*ash1-B-ns1 upf2Δ::URA3*
ZWY40	*ash1-C-ns upf2Δ::URA3*

(*continued*)

TABLE 1

(Continued)

Strain	Relevant genotype
ZWY47	*khd1Δ::KanMX4 she2Δ::URA3*
ZWY48	*ash1-A-ns1 khd1Δ::KanMX4 she2Δ::URA3*
ZWY49	*khd1Δ::KanMX4 she2Δ::URA3 upf1Δ::LEU2*
ZWY50	*ash1-A-ns1 khd1Δ::KanMX4 she2Δ::URA3 upf1Δ::LEU2*
ZWY51	*puf6Δ:KanMX4 she2Δ::URA3*
ZWY52	*ash1-A-ns1 puf6Δ:KanMX4 she2Δ::URA3*
ZWY53	*puf6Δ:KanMX4 she2Δ::URA3 upf1Δ::LEU2*
ZWY54	*ash1-A-ns1 puf6Δ:KanMX4 she2Δ::URA3 upf1Δ::LEU2*
JFY100	*leu2-Δ0 met15-Δ0 ura3-Δ04 lys3Δ puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY101	*ash1-A-ns1 leu2-Δ0 met15-Δ0 ura3-Δ04 lys3Δ puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY102	*upf1Δ::LEU2 met15-Δ0 ura3-Δ04 puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY103	*ash1-A-ns1 upf1Δ::LEU2 she2Δ::URA3 met15-Δ0 lys3Δ puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY104	*upf1Δ::LEU2 she2Δ::URA3 met15-Δ0 lys3Δ puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY106	*upf1Δ::LEU2 she2Δ::URA3 met15-Δ0 lys3Δ puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY107	*she2Δ::URA3 leu2-Δ0 puf6Δ::KanMX4 khd1Δ::KanMX4*
JFY109	*ash1-A-ns1 upf1Δ::LEU2 met15-Δ0 lys3Δ ura3-Δ04 puf6Δ::KanMX4 khd1Δ::KanMX4*

25 mM HEPES–KOH (pH 7.5), 150 mM KCl, 2 mM $MgCl_2$ 200 units/ml RNasin (Promega), 0.1% NP-40, 1 mM DTT, 0.2 μg/ml heparin, proteinase inhibitor cocktail (Sigma), and 2 mM vanadyl ribonucleoside (Sigma). Bacterial tRNA (0.2 μg) (Sigma) was used to saturate protein-G–agarose beads. IP was performed by preincubation of monoclonal anti-cmyc or anti-HA antibodies (Sigma) with protein-G–agarose at 4° overnight, followed by the addition of cell lysate at 4° for 2 hr. IP complexes were washed eight times, four with 500 μl of lysis buffer and four with 500 μl of lysis buffer containing 1 M urea.

TABLE 2

Plasmids

Plasmid	Description
pZW22	*CEN LEU2 ASH1*
pZW22-A-ns1	*CEN LEU2 ash1-A-ns1*
pZW22-A-ns2	*CEN LEU2 ash1-A-ns2*
pZW22-A-ms1	*CEN LEU2 ash1-A-ms1*
pZW22-B-ns1	*CEN LEU2 ash1-B-ns1*
pZW22-B-ns2	*CEN LEU2 ash1-B-ns2*
pZW22-B-ms1	*CEN LEU2 ash1-B-ms1*
pZW22-C-ms1	*CEN LEU2 ash1-C-ms1*
pZW22-C-ms2	*CEN LEU2 ash1-C-ms2*
pZW22-C-ns1	*CEN LEU2 ash1-C-ns1*
pC3319	*2μ LEU2 ASH1*
pC3319-A-ns1	*2μ LEU2 ash1-A-ns1*
pC3319-A-ns2	*2μ LEU2 ash1-A-ns2*
pC3319-A-ms1	*2μ LEU2 ash1-A-ms1*
pC3319-B-ns1	*2μ LEU2 ash1-B-ns1*
pC3319-B-ns2	*2μ LEU2 ash1-B-ns2*
pC3319-B-ms1	*2μ LEU2 ash1-B-ms1*
pC3319-C-ns1	*2μ LEU2 ash1-C-ns1*
pC3319-C-ms1	*2μ LEU2 adh1-C-ms1*
pZW18	*CEN HIS3 SHE2*
pXR192	*LEU2 ash1-mut-9myc*
pXR192-A-ns1	*LEU2 ash1-mut A-ns1*
pXR192-A-ns2	*LEU2 ash1-mu9 A-ns2*
pAA79	*CEN LEU2 UPF1*
pAA166	*CEN upf1Δ::LEU2*
pBSL321	*upf3Δ::URA3*
pRL199	*CEN LEU2 SHE2-myc6*

RNA recovery from IP and RT–PCR: Protein–RNA complexes were eluted from protein-G–agarose by incubation at 65° for 15 min in 100 μl of elution buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, and 1% SDS. RNA was extracted using phenol/chloroform, and the RNA was precipitated with ethanol and 150 mM sodium acetate (pH 5.2) overnight at −20°. The RNA pellet was washed with ice-cold 70% ethanol and treated with DNase (Ambion, Turbo DNA-free kit). RNA was quantified by two-step RT–PCR. Reverse transcription reactions were performed using the Superscript III cDNA synthesis kit (Invitrogen) or the high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR reactions were performed using the Taqman universal PCR kit (Applied Biosystems) on an ABI7900HT cycler. Gene-specific primers and Taqman probes were designed using PrimerExpress software. Background mRNAs present in mock experiments performed in the absence of antibodies were 2×10^{-3} less abundant relative to the same mRNAs recovered from IP experiments.

Statistical methods: Two-tailed *t*-tests assuming equal variance were performed and *P*-values were calculated to determine whether the relative levels of mRNA abundance were the same or different in pairwise sets of strains. The null hypothesis (H_0) was defined as the relative mutant *ash1* mRNA abundance equals relative wild-type *ASH1* mRNA abundance. ANOVA *F*-tests were performed and *P*-values were calculated to determine whether the relative fold changes in mRNA levels were the same or different in strains carrying *upf1Δ*, *upf2Δ*, or *upf3Δ*. The null hypothesis (H_0) was defined as the relative fold change in mutant *ash1* or wild-type *ASH1* mRNA abundance is equal in strains carrying *upf1Δ*, *upf2Δ*, or *upf3Δ*. Pearson's χ^2 and the corresponding *P*-value were calculated to determine whether deletions of genes coding for motor proteins and/or translational inhibitors affect the magnitude by which NMD influences the abundance of *ash1* nonsense mRNAs. The null hypothesis (H_0) was defined as the effect of inactivating NMD by deleting *UPF1* and the effect of deleting a gene coding for a motor protein and/or a translational inhibitor on *ash1* nonsense mRNA abundance are independent. For all of the statistical tests described above, a *P*-value of

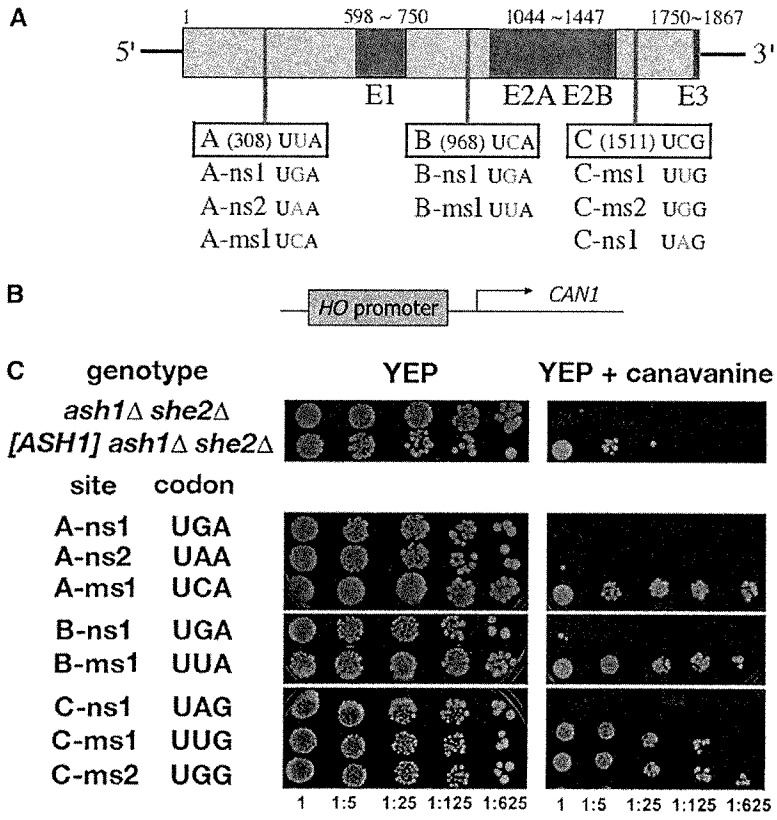


FIGURE 1.—Expression of nonsense and missense alleles of *ASH1*. (A) The map of *ASH1* shows the locations of missense and nonsense mutations. Purple boxes indicate the locations of regions (zip codes) required for She2p/RNA binding. Red lines and red letters indicate the locations and mutational changes at sites A, B, and C. Numbers refer to nucleotides in the *ASH1* open reading frame. (B) Structure of a reporter used to assay for the function of *ASH1* alleles. (C) The reporter was integrated in the genome as a replacement of the wild-type *CAN1* gene. Wild-type or mutant *ASH1* alleles were introduced into the reporter strain on a *CEN* plasmid. Transcriptional repression of the *HO* promoter by Ash1p confers canavanine resistance. Growth was assayed on plates containing synthetic defined medium plus 100 μg/ml canavanine using 1:5 serial dilutions of mid-log cultures.

0.05 was used as the standard cutoff. All experiments were repeated three times ($n = 3$). The results of the statistical analyses are described in the supplemental tables.

Cytological methods: Yeast strains W303a and AAY320 were transformed individually with 2μ plasmid pC3319 or derivatives of pC3319, where nonsense or missense mutations were introduced in the *ASH1* ORF. Transformants were grown to mid-log phase in synthetic liquid medium without leucine. Cells were fixed and *ASH1* mRNA localization was detected by fluorescent *in situ* hybridization with probes hybridizing to different parts of *ASH1* mRNA (LONG *et al.* 1997). Fifty anaphase cells with a visible *ASH1* signal were counted and scored for their localization phenotype. Results were based on two trials using independent transformants.

RESULTS

Nonsense mutations affect *ASH1* mRNA localization: To achieve localized protein expression, the translation of *ASH1* mRNA is repressed during transport, whereas the release of translational repression is required for proper anchoring at the bud tip as a prerequisite for local translation (CHARTRAND *et al.* 2002; GU *et al.* 2004; PAQUIN *et al.* 2007; DENG *et al.* 2008). Since premature termination of translation caused by a nonsense mutation might interfere with the release of repression and result in mRNA mislocalization, we performed experiments to assess the effects of nonsense mutations on localization. We analyzed nonsense mutations at three sites in *ASH1* (Figure 1A). Site A resides upstream of the E1, E2A, E2B, and E3 binding domains for She2p, whereas the other two sites, B and C, are located between the E1/E2A and E2B/E3 domains, respectively.

A reporter gene was used to monitor the ability of the alleles to produce functional Ash1p, a transcriptional repressor of the *HO* gene. The *HO* promoter was fused to *CAN1* (*HOp-CAN1*) (Figure 1B) (BOBOLA *et al.* 1996; JANSEN *et al.* 1996). In *ASH1 she2Δ* strains, *ASH1* mRNA mislocalizes, causing repression of *HOp-CAN1* in mother and daughter nuclei and leading to canavanine resistance. Wild-type and mutant alleles of *ASH1* were introduced into *she2Δ* strains carrying *HOp-CAN1* on a *CEN* plasmid. Growth was monitored in the presence of canavanine (Figure 1C). Strains carrying missense mutations at sites A, B, or C were resistant to canavanine, indicating that the missense alleles produced functional Ash1p. However, strains carrying nonsense mutations at the same three sites were sensitive to canavanine, indicating significantly reduced levels of functional Ash1p.

To examine mRNA localization in strains carrying the *ash1* alleles, Cy3-labeled fluorescent probes were used to detect the mRNAs *in situ* (MATERIALS AND METHODS). Anaphase cells were classified into three distinct localization phenotypes: crescent (localization at the bud tip), full bud (diffuse localization in the bud), and delocalized (diffuse localization in mother and bud) (Figure 2A). In asynchronous cultures of Nmd^+ strains carrying wild-type *ASH1*, the Cy3 signal was localized in a

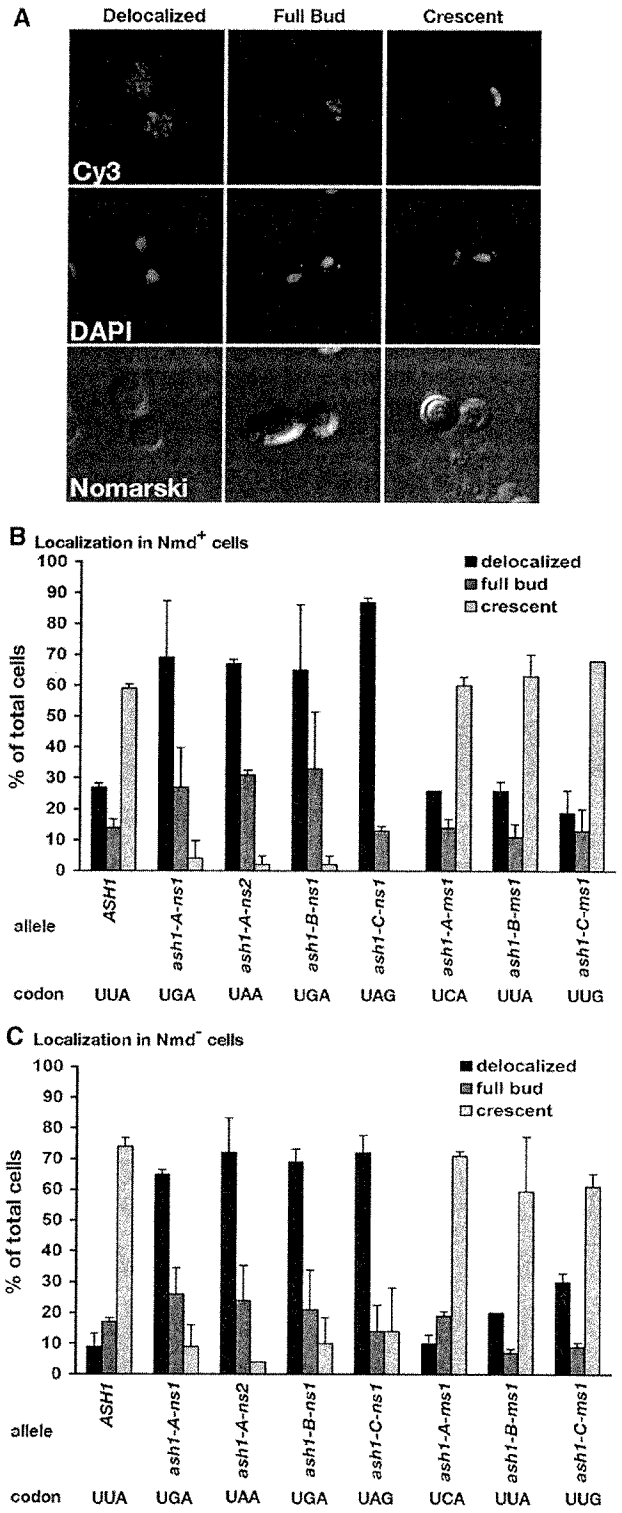


FIGURE 2.—Effects of nonsense and missense mutations on *ASH1* mRNA localization. (A) Nonsense codons affect the intracellular distribution of *ASH1* mRNA. Yeast strain W303a was individually transformed with 2μ plasmids carrying different alleles of *ASH1* and analyzed by fluorescent *in situ* hybridization (MATERIALS AND METHODS; LONG *et al.* 1997). In cells classified as "delocalized," *ASH1* mRNA was symmetrically distributed between the mother cell and the bud. In cells classified as "full bud," *ASH1* mRNA was distributed throughout the daughter cell. In cells classified as "crescent," *ASH1* mRNA formed a tight crescent at the bud tip. (B) Quantification of the effects of premature termination codons on *ASH1* mRNA localization. The phenotype for 50 late-anaphase cells in A expressing mutant alleles of *ASH1* mRNA were counted and classified as localized, full bud, or delocalized. Two independent trials were performed.

crescent at the bud tip in ~60% of cells (Figure 2B). Nonsense mutations at all three sites caused a reduction to <5% of anaphase cells showing a crescent localization pattern, whereas the percentage of cells showing a delocalized signal rose significantly. Missense mutations at sites A, B, and C had no discernible effects on localization. The results indicate that shifts from the crescent to the delocalized pattern in cells carrying the nonsense mutations are most likely due to premature termination of translation.

In addition to the increased percentage of cells showing the delocalized signal, nonsense mutations at sites A and B caused a modest but statistically significant increase in the percentage of anaphase cells showing the full-bud mislocalization pattern. Together, the results suggest that the corresponding nonsense mRNAs were transported to the bud tip, but failed to anchor. The changes in localization caused by the nonsense mutation at site C also indicate a failure to anchor, but the effects were more dramatic. Almost 90% of cells showed a delocalized pattern. In She2p/RNA-binding experiments described in a later section, we show that nonsense mutations at sites A and B cause the mRNAs to mislocalize while remaining bound to She2p. The mRNAs are therefore still tethered to actin cables. The nonsense mutation at site C causes release of the mRNA from She2p. The mRNA probably exhibits more pronounced mislocalization because it is no longer tethered to actin cables.

The changes in localization caused by the nonsense mutations could affect the ratio of translationally repressed mRNAs engaged in transport and the translatable mRNAs that are anchored at the bud tip. Since NMD requires translation, a shift toward more translationally repressed mRNA at the expense of translatable mRNA could reduce the overall sensitivity of the nonsense mRNAs to NMD. Furthermore, NMD itself might affect localization. When the nonsense mutations were examined in Nmd⁻ strains, the patterns of mislocalization were similar to those observed for Nmd⁺ strains (Figure 2C). Given limits on information that can be gained from cytological analysis, additional approaches described below were pursued to assess the potential impact of NMD on translation termination, decay, and localization of *ash1* nonsense mRNAs.

Changes in mRNA levels associated with premature translation termination and NMD: Since the accelerated decay of nonsense mRNAs caused by NMD is a direct consequence of premature termination of translation, we anticipated that the accumulation of missense

TABLE 3

Fold changes in mRNA abundance in *UPF1* and *upf1Δ* strains

NMD	*ASH1* allele	Fold change *ash1/ASH1*	*P*[a]	Effect on abundance	
				ash1 allele	NMD
UPF1	*ASH1*[b]	1.00 ± 0.08	1	None	
upf1Δ	*ASH1*[b]	0.92 ± 0.25	1		
upf1Δ/UPF1		0.92 ± 0.25	0.68		None
UPF1	*ash1-A-ns1*[b]	0.98 ± 0.08	0.87	None	
upf1Δ	*ash1-A-ns1*[b]	4.07 ± 0.16	*0.00009*		
upf1Δ/UPF1		4.15 ± 0.16	*0.0001*		*Increase*
UPF1	*ash1-A-ns2*[c]	0.97 ± 0.42	0.91	None	
upf1Δ	*ash1-A-ns2*[c]	2.84 ± 0.55	*0.0003*		
upf1Δ/UPF1		2.93 ± 0.57	*0.002*		*Increase*
UPF1	*ash1-A-ms1*[c]	0.72 ± 0.09	0.12	None	
upf1Δ	*ash1-A-ms1*[c]	0.82 ± 0.20	0.25		
upf1Δ/UPF1		1.14 ± 0.28	0.42		None
UPF1	*ash1-B-ns1*[b]	1.54 ± 0.23	*0.02*	*Increase*	
upf1Δ	*ash1-B-ns1*[b]	1.52 ± 0.10	*0.01*		
upf1Δ/UPF1		0.99 ± 0.06	0.92		None
UPF1	*ash1-B-ms1*[c]	1.21 ± 0.46	0.48	None	
upf1Δ	*ash1-B-ms1*[c]	0.89 ± 0.12	0.06		
upf1Δ/UPF1		0.74 ± 0.26	0.23		None
UPF1	*ash1-C-ns1*[b]	1.87 ± 0.26	*0.0008*	*Increase*	
upf1Δ	*ash1-C-ns1*[b]	1.73 ± 0.28	*0.005*		
upf1Δ/UPF1		0.93 ± 0.15	0.70		None
UPF1	*ash1-C-ms1*[c]	1.44 ± 0.67	0.27	None	
upf1Δ	*ash1-C-ms1*[c]	0.85 ± 0.28	0.25		
upf1Δ/UPF1		0.59 ± 0.19	0.15		None
UPF1	*ash1-C-ms2*[c]	1.18 ± 0.44	0.53	None	
upf1Δ	*ash1-C-ms2*[c]	0.93 ± 0.52	0.33		
upf1Δ/UPF1		0.79 ± 0.44	0.49		None

[a] Statistical analyses were performed using two-tailed *t*-tests (MATERIALS AND METHODS). H_0: mutant *ash1* mRNA abundance equals wild-type *ASH1* mRNA abundance. H_1: mutant *ash1* mRNA abundance does not equal wild-type *ASH1* mRNA abundance. *P*-values <0.05 (italic) indicate a change in abundance.

[b] Experiments were performed by expressing *ash1* alleles that were integrated at the *ASH1* locus by gene replacement.

[c] Experiments were performed by expressing *ash1* alleles from a centromeric plasmid.

mRNAs would be unaffected by the loss of NMD. Consistent with expectation, missense mutations at sites A, B, or C (*ash1-A-ms1*, *ash1-B-ms1*, *ash1-C-ms1*, and *ash1-C-ms2*) had no effect on mRNA abundance either in Nmd$^+$ strains or in Nmd$^-$ strains carrying *upf1Δ*, a deletion that inactivates NMD (Table 3).

Since NMD accelerates the decay of nonsense mRNAs, the accumulation of nonsense mRNAs are typically reduced compared to the corresponding wild-type mRNA, whereas the wild-type level is restored when NMD is inactivated. However, the levels of *ash1* nonsense mRNAs could deviate from expectation in the event of a shift favoring a higher proportion of translationally repressed, NMD-insensitive mRNAs. The cytological evidence showing that the nonsense mRNAs cause mislocalization was suggestive of this possibility.

When transcripts produced from the nonsense alleles were analyzed in Nmd$^+$ strains, the reduced levels typical of most nonsense mRNAs were not observed (Table 3). Instead, we found that the relative levels of *ash1-A-ns1* and *ash1-A-ns2* mRNAs were indistinguishable from the wild-type *ASH1* mRNA. In Nmd$^-$ strains carrying *upf1Δ*, these nonsense mRNAs were detected at a 3- to 4-fold higher level compared to *ASH1* mRNA. The *ash1-B-ns1* and *ash1-C-ns1* mRNAs behaved differently. In Nmd$^+$ strains, they were 1.5- to 2-fold more abundant than *ASH1* mRNA. In Nmd$^-$ strains, the same excess accumulation was observed as in Nmd$^+$ strains but without any further changes that could be attributed to the inactivation of NMD.

To summarize the data, we found that nonsense mutations at all three sites cause increased mRNA

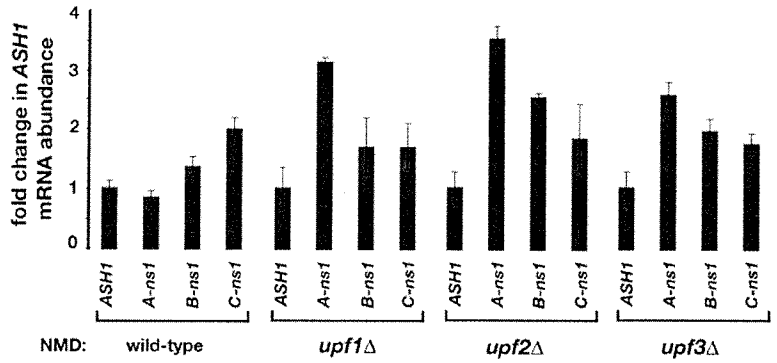


FIGURE 3.—Accumulation and decay of *ash1* nonsense mRNAs. The relative fold changes in mRNA levels of the nonsense mRNAs compared to *ASH1* mRNA are shown. The accumulation of *ASH1* mRNA was compared with the accumulation of representative *ash1* nonsense mRNAs in strains carrying null alleles of *UPF1*, *UPF2*, and *UPF3*. The mRNAs were detected by Northern blotting using a probe that anneals to *ASH1* mRNA. The RNA levels were normalized to *RPS3* mRNA, which is not affected by NMD (GUAN *et al.* 2006).

accumulation, but the only mRNAs that responded to the inactivation of *UPF1* were those carrying nonsense mutations at site A. Similar experiments were performed using congenic sets of strains carrying deletions of *UPF1*, *UPF2*, or *UPF3*. The same results were obtained regardless of which *UPF* gene was deleted (Figure 3, Table 4). The NMD-dependent increases in mRNA abundance observed for mutations at site A are therefore most likely due to the inactivation of NMD rather than to the loss of function of a specific *UPF* gene.

Transcript selection and decay of *ash1-A-ns1* mRNA: The phenotypes described above for nonsense mutations at site A deviate from what has been observed for the typical nonsense mutation in the typical gene where mRNA abundance is usually reduced due to NMD. Since nonsense mutations at site A produced transcripts that exhibit NMD-sensitive increases in accumulation, we performed additional experiments to confirm a role for NMD and to explain the underlying reasons for the deviations from expectation.

We asked whether Upf1p preferentially binds to the *ash1-A-ns1* nonsense transcript, which is a diagnostic indicator of NMD targeting (JOHANSSON *et al.* 2007). Using IP/quantitative RT–PCR (MATERIALS AND METHODS), we found that the amount of *ash1-A-ns1* mRNA associated with Upf1p was more than six-fold higher compared to *ASH1* mRNA (Figure 4B). The IP was performed with lysates from strains that carried chromosomal *upf1Δ* and an allele of *UPF1* on a *CEN* vector that produces a functional epitope-tagged product (HA-Upf1p). The ratios of *ASH1* and *ash1-A-ns1* mRNAs were calculated relative to *RDR1* mRNA.

If *ash1-A-ns1* mRNA is targeted by NMD, the nonsense transcript should become more stable when NMD is inactivated and less stable compared to the wild-type mRNA in Nmd$^+$ strains. The decay rates were examined using the temperature-shift method for transcriptional shutoff followed by Northern blotting at intervals following shutoff (MATERIALS AND METHODS). Biphasic decay was observed for both the wild-type and the nonsense mRNA irrespective of NMD (Figure 4C). The initial phase was characterized by rapid mRNA disappearance (phase I) followed by a phase of apparent stability (phase II). The phase I decay rates for *ASH1* mRNA in Nmd$^+$ and Nmd$^-$ strains were indistinguishable (2.8 ± 0.8 and 2.7 ± 0.7 min, respectively), indicating that NMD had no effect on the decay of the wild-type mRNA. The nonsense mRNA was stabilized in Nmd$^-$ strains. Phase I decay rates for *ash1-A-ns1* mRNA in Nmd$^+$ and Nmd$^-$ strains were 3.6 ± 0.1 and 7.2 ± 2.5 min, respectively.

An unexpected result was observed when the decay of the wild-type and nonsense mRNAs were compared. The phase I decay rates were statistically similar (2.8 ± 0.8 and 3.6 ± 0.1, respectively) in Nmd$^+$ strains, but the nonsense mRNA should have a faster decay rate if it is targeted by NMD. By comparison, the accumulation of the nonsense mRNA was higher than wildtype in Nmd$^+$ strains, but NMD targeting should cause it to be lower.

One possible explanation for the deviation from expectation is suggested by the changes in localization described above that are associated with nonsense mutations. If the mutations cause a shift toward translationally repressed mRNA at the expense of translat-

TABLE 4

Relative mRNA abundance in Nmd$^-$ strains carrying different *upf* gene deletions

Allele	*UPF1*	*upf1Δ*	*upf2Δ*	*upf3Δ*	*P*[a]
ASH1	1.00 ± 0.10	1.00 ± 0.31	1.00 ± 0.23	1.00 ± 0.23	0.46
ash1-A-ns1	0.86 ± 0.09	2.96 ± 0.06	3.32 ± 0.18	2.44 ± 0.19	0.36
ash1-B-ns1	1.33 ± 0.14	1.64 ± 0.44	2.41 ± 0.05	1.88 ± 0.17	0.72
ash1-C-ns1	1.92 ± 0.16	1.64 ± 0.36	1.77 ± 0.52	1.68 ± 0.15	0.52

[a] Data were evaluated using *F*-tests (MATERIALS AND METHODS).

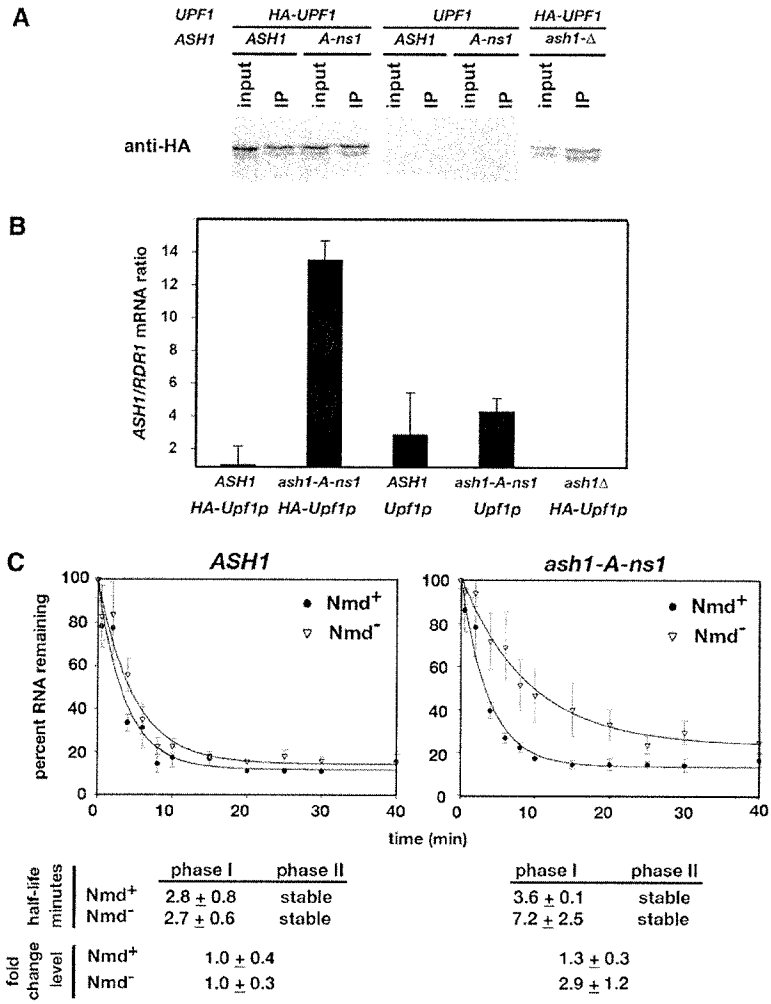


FIGURE 4.—Co-immunoprecipitation of *ASH1* and *ash1-A-ns1* mRNA with Upf1p. (A) Anti-HA antibodies immunoprecipiate HA-UPF1 as detected by Western blotting of a post-IP lysate. (B) The relative amounts of *ASH1* and *ash1-A-ns1* mRNAs recovered in the post-IP lysates were assayed quantitatively using real-time RT–PCR. Relative RNA amounts were normalized to *RDR1* RNA. (C) The rates of decay of *ASH1* and *ash1-A-ns1* mRNAs were determined by measuring RNA levels after inhibition of transcription using the temperature-shift method in strains carrying *rpb1-1* (MATERIALS AND METHODS). Real-time RT–PCR was used to quantify the relative amounts of RNA. 18s rRNA was used as loading control. The graphs represent the relative amounts of mRNA remaining following the inhibition of transcription at t_0. The error bars show standard deviations based on three trials.

able mRNA, a greater proportion of the nonsense mRNA pool would be insensitive to NMD as compared to the pool of wild-type mRNA. Since the estimated phase I decay rates are composite averages of the decay rates of any subpopulations that are present, an increased pool of translationally repressed, NMD-insensitive mRNA would cause the composite decay rate of the nonsense mRNA to appear artificially higher than the decay rate of the wild-type mRNA. An increase in the proportion of more stable, NMD-insensitive mRNA might also affect phase II decay rates, but phase II is more difficult to assess because of the potential contribution of low level, residual transcription caused by incomplete inhibition of transcription. Residual transcription contributes in a minor way to phase I decay, but is more significant in phase II because low-level, ongoing transcription in phase II represents a higher proportion of the mRNAs remaining after transcriptional shutoff. Residual transcription is typically not >10% of the total mRNA (LELIVELT and CULBERTSON 1999; GUAN *et al.* 2006).

Relative pool size of She2p-bound mRNA: To understand the underlying causes of deviations from expectation as described above, we examined the transcript pool that binds to She2p. Since these transcripts are mostly if not entirely engaged in transport via actin cables, the She2-bound mRNA pool most likely corresponds to the translationally repressed, NMD-insensitive pool. If the relative proportion of nonsense mRNA bound to She2p increases compared to wild type, this might explain the unexpectedly high levels of accumulation observed for nonsense mRNAs and the longer-than-expected half-lives.

The relative amounts of She2p bound to wild-type *ASH1* and three *ash1* nonsense transcripts were determined by IP/quantitative RT–PCR. Lysates were prepared for IP from Nmd$^+$ and Nmd$^-$ strains carrying an allele of *SHE2* that produces a functional epitope-tagged product (She2p-cmyc; see MATERIALS AND METHODS). The amount of RNA recovered by RT–PCR was normalized to a control mRNA, *IST2*, which localizes on actin cables but is not affected by NMD (LELIVELT and CULBERTSON 1999; GUAN *et al.* 2006).

In Nmd$^+$ strains, the relative amount of *ash1-A-ns1* mRNA that copurified with She2p-cmyc was increased

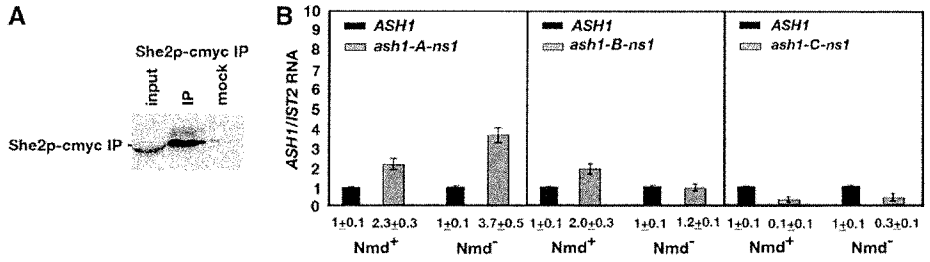


FIGURE 5.—Co-immunoprecipitation of *ASH1* and *ash1-A-ns1* mRNA with She2p. (A) Anti-cmyc antibodies immunoprecipitate She2p-cmyc as detected by Western blotting of a post-IP lysate. (B) The relative amounts of *ASH1* and *ash1-A-ns1* mRNAs recovered in the post-IP lysates from Nmd$^+$ and Nmd$^-$ strains were assayed quantitatively using quantitative RT–PCR. Relative RNA amounts were normalized to *IST2* RNA, which is unaffected by the inactivation of *UPF* genes.

2.3- ± 0.3-fold compared to the wild-type mRNA (Figure 5A) even though both transcripts were similar in total abundance when assayed by Northern blotting (Table 3, Figure 3). In Nmd$^-$ strains, the relative amount of She2p-bound *ash1-A-ns1* mRNA was increased 3.7- ± 0.5-fold compared to the wild-type mRNA with a corresponding 3- to 4-fold increase in total *ash1-A-ns1* mRNA abundance (Table 3, Figure 3). Since both premature termination of translation at the A site and loss of NMD contribute to an increased proportion of She2p-bound mRNA, the results support a model developed further in the DISCUSSION in which a higher proportion of the nonsense transcript is protected from NMD by translational repression, which causes distortions in the expected effects of NMD.

In Nmd$^+$ strains, the relative amount of She2p-bound *ash1-B-ns1* nonsense mRNA was twice that of *ASH1* mRNA (Figure 5B) compared with a 1.5-fold increase in total mRNA (Figure 3, Table 3). However, in Nmd$^-$ strains, the relative amount of She2-bound *ash1-B-ns1* was the same as wild-type mRNA, possibly because the loss of NMD has no effect on the total abundance of *ash1-B-ns1* mRNA (Table 3, Figure 3). Thus, premature translation termination caused by the *ash1-B-ns1* and *ash1-A-ns1* mutations have similar effects on She2-bound pool size, but the two nonsense transcripts differ in their sensitivity to NMD, possibly due to the binding of She2p at domain E1 located between sites A and site B (Figure 1A). She2p bound at E1 might affect the ability of ribosomes to reach the termination codon at site B.

The *ash1-C-ns1* mutation differed dramatically from the nonsense mutations at sites A and B. Although total *ash1-C-ns1* mRNA increased twofold compared to the wild-type mRNA (Figure 3, Table3), the relative amount of mRNA bound to She2p-cmyc was reduced by 90% (Figure 5B). The mislocalization of *ash1-C-ns1* nonsense mRNA (Figure 2) presumably differs from the mislocalization of mRNAs containing nonsense mutations at sites A and B because the *ash1-C-ns1* nonsense mRNA is no longer bound to the actin cytoskeleton, whereas nonsense mRNAs containing mutations at sites A and B show increased binding to the actin cytoskeleton.

Behavior of *ash1* nonsense transcripts in transport-defective mutants: To see if disruption of the transport machinery affects the abundance and decay of *ash1* nonsense transcripts, the relative levels of *ash1* nonsense mRNAs were examined in Nmd$^+$ and Nmd$^-$ strains carrying *she2Δ* (supplemental Table S1). In Nmd$^+$ strains, the relative levels of wild-type, *ash1-A-ns1*, and *ash1-B-ns1* nonsense mRNAs were significantly reduced compared to *SHE2* strains. The *ash1-C-ns1* mRNA was modestly elevated, but with marginal statistical significance ($P = 0.049$). In Nmd$^-$ strains, the *ash1-A-ns1* mRNA was equally sensitive to NMD in both *SHE2* and *she2Δ* strains. The *ash1-C-ns1* mRNA was unaffected by NMD in both *SHE2* and *she2Δ* strains.

Although the *ash1-B-ns1* mRNA level was not affected by NMD in *SHE2* strains (Table 3), a twofold increase in abundance was observed in *she2Δ* Nmd$^-$ strains (supplemental Table S1). Decay rates were compared in *SHE2 UPF1*, *she2Δ UPF1*, *SHE2 upf1Δ*, and *she2Δ upf1Δ* strains (Figure 6). In all four strains, biphasic decay was observed, indicating the existence of at least two pools of transcripts that decay at different rates. In *SHE2* strains, the *ash1-B-ns1* mRNA was insensitive to NMD (Figure 6A). However, in *she2Δ* strains, it was NMD sensitive. The phase I half-lives were 1.7 ± 0.1 and 2.6 ± 0.6 min in the Nmd$^+$ and Nmd$^-$ strains, respectively, which corresponds to a 1.5-fold, statistically significant difference. Since two pools of differentially decaying transcripts were detected in *SHE2* strains and since the She2-bound pool was eliminated in the *she2Δ* strains, the NMD-insensitive pool in *she2Δ* strains consists of transcripts that are no longer tethered to the actin cytoskeleton but that remain translationally repressed.

As an alternative approach to disrupt transport, we made use of a previously reported allele called *ash1-MUT*, which contains multiple mutations in the zip codes that prevent mRNA binding to She2p, but without changing the amino acid sequence of the protein product. The *ash1-MUT* mRNA is delocalized because it cannot bind to She2p and fails to tether to actin cables (CHARTRAND *et al.* 2002). The nonsense mutations in *ash1-A-ns1* and *ash1-A-ns2* were combined with *ash1-MUT* to produce *ash1-MUT-A-ns1* and *ash1-MUT-A-ns2* and then integrated at the *ASH1* locus by gene replacement in congenic Nmd$^+$ and Nmd$^-$ strains. When mRNA levels were assayed by Northern blotting, we

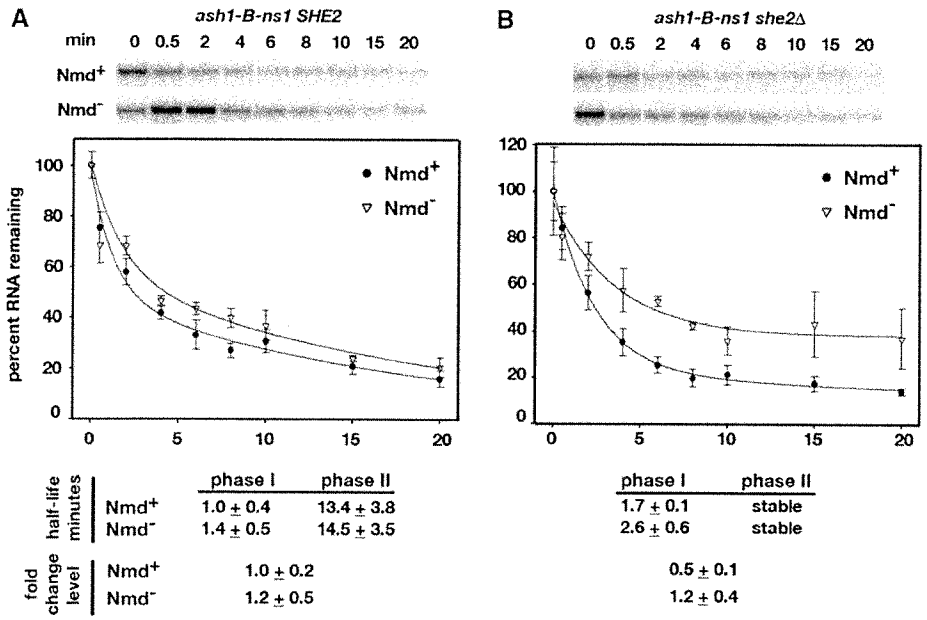


FIGURE 6.—Effects of *she2Δ* on the rate of *ash1-B-ns1* mRNA decay in Nmd$^+$ and Nmd$^-$ strains. The decay rate of *ash1-B-ns1* mRNAs was determined by measuring the disappearance of mRNA detected by Northern blotting following the inhibition of transcription with 15 μg/ml thiolutin (MATERIALS AND METHODS). (A) Decay rates in Nmd$^+$ and Nmd$^-$ strains that carry *SHE2*. (B) Decay rates in Nmd$^+$ and Nmd$^-$ strains that carry *she2Δ*. Standard error was based on three trials.

found that the inactivation of NMD had the same effect on mRNA accumulation regardless of whether tethering to the transport system was disrupted by deletion of *SHE2*, as described above, or by disruption of She2p binding at the zip codes (supplemental Table S2).

We wanted to know whether a more general disruption of transport affects the behavior of *ash1* nonsense mRNA. The relative levels of wild-type *ASH1* and *ash1-A-ns1* nonsense mRNAs were determined by Northern blotting in strains carrying deletions of genes required for transport, including *she1Δ* (type V myosin motor protein), *she3Δ* (actin–myosin adaptor protein), *she4Δ* (regulator of She1p), and *she5Δ* (actin filament assembly). For each set of *sheΔ* strains, the inactivation of NMD caused a three- to fivefold increase in *ash1-A-ns1* mRNA abundance compared to *ASH1* mRNA abundance (supplemental Table S3). Chi-square tests indicated that the magnitudes of change caused by the inactivation of NMD were similar regardless of which *SHE* gene was deleted (supplemental Table S4). The effects of deleting these genes were similar to effects of *she2Δ* or the mutations in *ash1-MUT* that prevent tethering of the mRNA to the transport system. Overall, the results indicate that disruption of transport did not cause increased sensitivity of *ash1-A-ns1* nonsense mRNA to NMD. These results might be explained if mRNAs that are not tethered to the actin cytoskeleton remain translationally repressed.

Behavior of *ash1-A-ns1* nonsense mRNA in the absence of translational repressors: The protein products of *PUF6*, *KHD1*, and *LOC1* have been implicated in mediating translational repression of *ASH1* mRNA during transport (LONG *et al.* 2001; IRIE *et al.* 2002; GU *et al.* 2004). The effects of deleting genes coding for translational repressors were investigated in Nmd$^+$ and Nmd$^-$ strains expressing *ash1-A-ns1* mRNA (supplemental Tables S4–S6) to see if relief of translational repression might cause an increased proportion of transcripts that are sensitive to NMD. We found that *puf6Δ* caused an overall reduction in the abundance of *ash1-A-ns1* mRNA, but the effect was unrelated to NMD. The reduction could be due to either direct or indirect effects of Puf proteins on mRNA stability (WICKENS *et al.* 2002). Single deletions of *KHD1* or *LOC1* had no significant effect on mRNA abundance, and no increase in sensitivity to NMD was observed as the result of deleting these translational repressors one at a time. These experiments were extended by analyzing *khd1Δ* and *puf6Δ* double deletions in combination with a *she2Δ* deletion to see if *ash1-A-ns1* mRNAs that are not tethered to the actin cytoskeleton are more sensitive to NMD in the absence of translational repressors. Once again, no increase in sensitivity was observed (supplemental Table S6).

One possible explanation for the results described above is that the translational repressors have redundant effects on translation. Maximal sensitivity to NMD might occur only in transport-defective mutants where the genes for translational inhibitors are simultaneously deleted. To test this, *ash1-A-ns1* mRNA was analyzed in *she2Δ* strains carrying deletions of *UPF1*, *KHD1*, and *PUF6* in multiple combinations. When mRNA levels were compared, we found, as expected from previous results, that disruption of NMD caused increased accumulation of *ash1-A-ns1* mRNA (Figure 7A). In Nmd$^+$ strains, a trend toward higher accumulation of *ash1-A-ns1* mRNA was observed with the highest level found in a strain simultaneously deleted for *UPF1*, *KHD1*, *PUF6*, and *SHE2*.

To assess whether changes in mRNA abundance in the quadruple mutant reflect underlying changes in mRNA half-life, we measured the kinetics of decay of

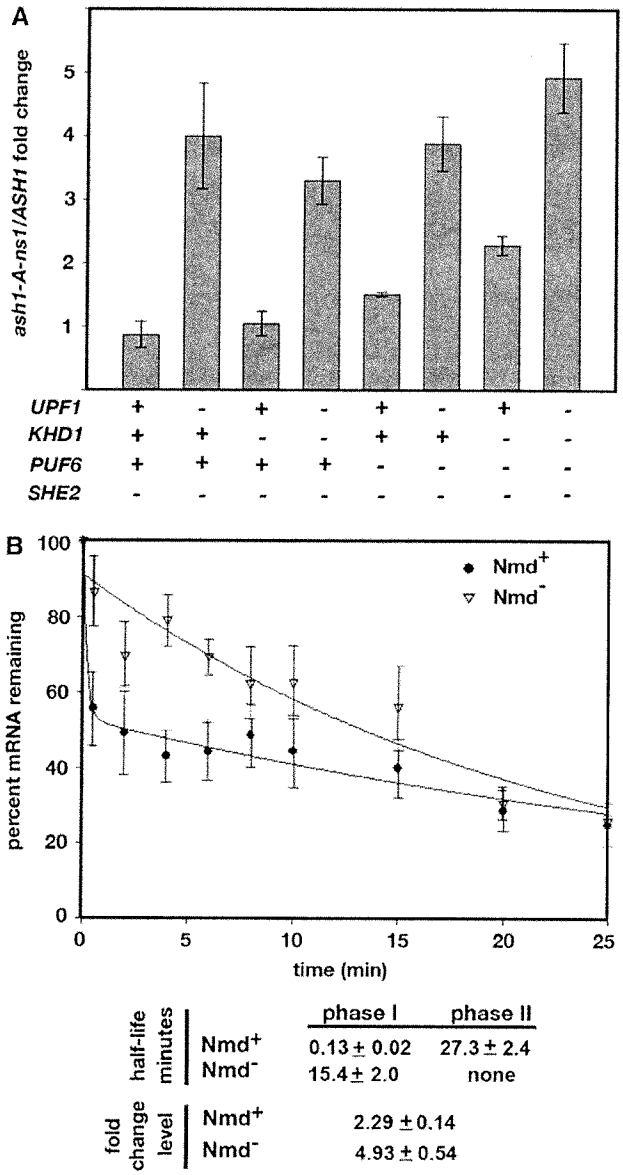


FIGURE 7.—Accumulation and decay of *ash1-A-ns1* mRNA in *she2Δ* strains lacking inhibitors of *ASH1* translation. (A) Levels of accumulation were compared by Northern blotting. (B) The decay of *ash1-A-ns1* mRNA was determined in Nmd$^+$ and Nmd$^-$ strains carrying *upf1Δ*, *khd1Δ*, *puf6Δ*, and *she2Δ* as described in Figure 6 and MATERIALS AND METHODS.

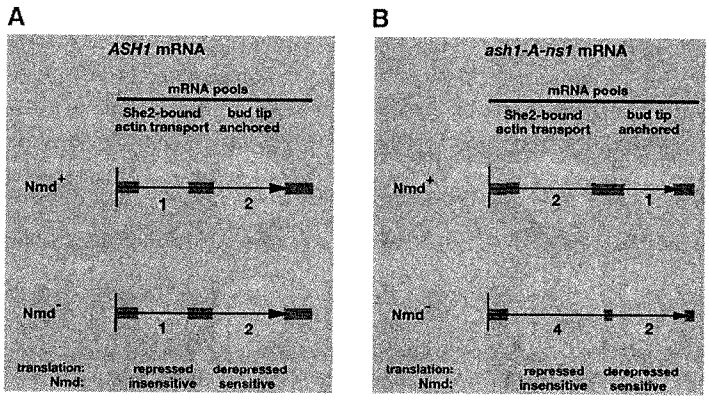
ash1-A-ns1 mRNA in Nmd$^+$ and Nmd$^-$ strains carrying simultaneous deletions of *KHD1*, *PUF6*, and *SHE2* (Figure 7B). The nonsense mRNA decayed with biphasic kinetics in the Nmd$^+$ strain. The phase I decay rate was extremely rapid with an estimated half-life of 0.13 ± 0.02 min. The kinetics of decay also show the presence of a more slowly decaying pool of mRNAs with a half-life of 27 ± 2.4 min. In the Nmd$^-$ strain, decay was monophasic. A dramatic stabilization was observed in which the overall half-life was 15.4 ± 2.0 min. A NMD-insensitive mRNA subpopulation corresponding to phase II could still be present in the Nmd$^-$ strain, but might be obscured by the predominant NMD-sensitive subpopulation. Compared with previous observations, these results suggest that the *ash1-A-ns1* nonsense transcript is hypersensitive to NMD when it cannot tether to the transport system and when it can be more efficiently translated in the absence of the Khd1p and Puf6p translational repressors.

DISCUSSION

To our knowledge, there have been no studies that address whether particular classes of transcripts are immune to or sequestered from the effects of NMD. We studied *ASH1* mRNA to assess how mRNAs that are transported by the actin cytoskeleton prior to translation are affected by blocks in translation and whether the mRNAs are sensitive or immune to NMD. According to previously proposed models (CHARTRAND *et al.* 2002), tight regulation exists between two temporally incompatible events: translation and the transport of mRNAs tethered to the transport machinery through the binding of She2p to the mRNAs. Our data support a model in which full-length translation of *ASH1* mRNA is an integral part of the maturation pathway. Blocks in translation cause mislocalization. Furthermore, *ash1* nonsense mRNAs are prone to NMD, but sensitivity to NMD depends on the position of the mutation.

The *ashA-ns1* nonsense mRNA accumulated and responded to loss of NMD in a manner atypical of transcripts encoded by nonsense alleles of other genes. Typically, nonsense mRNA abundance is reduced compared to the corresponding wild-type mRNA. The reduction is caused by an acceleration of the mRNA half-life due to targeting of the mRNA by NMD. When NMD is inactivated, the abundance rises back to the same level as the wild-type mRNA (LEEDS *et al.* 1992). By contrast, the wild-type *ASH1* and nonsense *ash1-A-ns1* mRNAs were equally abundant in Nmd$^+$ strains. When NMD was inactivated, the *ash1-A-ns1* nonsense mRNA was two- to fourfold more abundant than wild-type *ASH1* mRNA.

An explanation for the atypical behavior comes from the finding that the relative proportion of *ash1-A-ns1* mRNA bound to She2p, and therefore tethered to the actin cytoskeleton, rose twofold in Nmd$^+$ strains and fourfold in Nmd$^-$ strains. These results reflect an anchoring defect caused by the nonsense mRNA that is accentuated when NMD is inactivated. The results are summarized in Figure 8. The underlying cause of deviations in the expected behavior of *ash1-A-ns1* mRNA appears to be related to the division of *ASH1* mRNA into two pools. One pool, which is bound to She2p, is engaged in transport. These mRNAs are translationally repressed. Since NMD requires translation, the She2p-bound pool is insensitive to NMD. The other pool is anchored at the bud tip. These mRNAs are translationally derepressed and sensitive to NMD. This could be



C

Genotype NMD	ASH1	translation repressed : derepressed relative amounts		total amount
+	WT	1	2	3
+	A-ns1	2	1	3
–	WT	1	2	3
–	A-ns1	4	2	6

FIGURE 8.—Dual-pool model. Nonsense mutations at the A site (Figure 1) cause a change in the relative proportion of two pools of mRNA: (1) She2p-bound mRNAs, which are tethered to the actin cytoskeleton, are engaged in transport, and are translationally repressed and NMD insensitive and (2) mRNAs anchored at the bud-tip cortex, which are translatable and sensitive to NMD. The numbers in the circles in A and B represent the relative pool sizes on the basis of experimental evidence shown in Figure 5. The same relative ratios are shown in tabular form in C. Changes in the relative pool sizes due to premature termination at site A and to the inactivation of NMD are consistent with data presented in the text.

explained by changes in the relative sizes of the two pools caused by premature termination of translation and are further accentuated by the inactivation of NMD.

According to the model (Figure 8), impaired anchoring caused by premature termination at the A site causes a change in the relative sizes of the two pools in Nmd$^+$ strains, favoring an increase in the size of the transport pool at the expense of the anchored pool. This reduces the proportion of mRNAs that are sensitive to NMD. Because of the shift toward NMD-insensitive mRNAs, the measured half-life of *ash1-A-ns1* (Figure 4) appears to be similar to wild-type *ASH1* mRNA, but in reality the shift masks more rapid decay of the NMD-sensitive pool. The data on accumulation, decay, and the relative binding of *ash1-A-ns1* mRNA with She2p are predictable outcomes of the model. Most notably, twice as much *ash1-A-ns1* mRNA is bound to She2p compared to *ASH1* mRNA in Nmd$^+$ strains, whereas four times as much is bound in Nmd$^-$ strains (Figure 5). Furthermore, the total abundance of the nonsense and wild-type mRNAs are the same in Nmd$^+$ strains, but in Nmd$^-$ strains the nonsense mRNA is more abundant (Figure 3).

In Nmd$^-$ strains, mRNAs in the anchored pool are more stable, which increases the size of the pool. If anchoring of the nonsense mRNA is a rate-limiting bottleneck for entry into this pool, slower decay of the anchored pool might slow entry into the pool and cause a rise in the size of the She2p-bound pool. Alternatively, in the absence of Upf1p, less efficient termination might also slow entry into the anchored pool since Upf1p is known to promote efficient termination of translation in conjunction with the release factors eRF1 and eRF3 as a prerequisite for decapping and decay (WANG *et al.* 2001). In either case, our data support the idea that anchoring depends not only on translation but also on the rate at which anchored mRNAs are degraded.

To reveal the full potential of NMD to degrade *ash1-A-ns1* mRNA, we disrupted the transport system by deleting five different *SHE* genes and by analyzing nonsense alleles in which the *ash1-A-ns1* and *ash1-A-ns2* mutations were combined with multiple mutations in the zip codes to prevent the binding of She2p. Although none of the mutations had any added effect on sensitivity to NMD by themselves or in combination with single deletions of genes coding for translational repressors of *ASH1*, we found that *ash1-A-ns1* mRNA was hypersensitive to NMD in *she2Δ* strains carrying simultaneous deletions of *PUF6* and *KHD1*. The rapid decay in the triple-deletion strain demonstrates the full efficacy of NMD-mediated degradation of *ash1-A-ns1* mRNA, but this rapid decay occurs only when the mRNA is in an actin-detethered, translationally derepressed pool that does not normally exist.

The nonsense mutations at sites B and C differ from site A since the latter is located upstream of all She2p-binding domains whereas the former are located downstream of one or more of the binding domains. Like the mutations at site A, mutations at sites B and C cause increased mRNA abundance relative to wild type in Nmd$^+$ strains, but unlike the mutations at site A, the corresponding mRNAs are insensitive to the inactivation of NMD in otherwise wild-type strains. The nonsense mutation at site B causes a mislocalization phenotype similar to nonsense mutations at site A, but the mutation at site B confers more severe mislocalization. Like *ash1-A-ns1*, the *ash1-B-ns1* mutation causes a shift toward increased amounts of mRNA bound to She2p in Nmd$^+$ strains, but unlike *ash1-A-ns1*, no further increase was observed when NMD was inactivated, consistent with its insensitivity to NMD. Reduced binding to She2p was observed for a nonsense mutation at site C in both Nmd$^+$ and Nmd$^-$ strains.

Most importantly, the *ash1-B-ns1* nonsense mRNA, which was insensitive to NMD in *SHE2* strains, became sensitive in *she2Δ* strains as evidenced by changes in mRNA abundance and decay rate in Nmd$^-$ strains. The difference in phenotypes between mutations at sites A and B might be explained by their locations relative to positions of the binding sites for She2p. The sequence of events at the time of anchoring is unknown. However, if repression of translation initiation is relieved before the mRNA is released from binding to She2p, translation could initiate and proceed unimpeded to site A where translation terminates. However, ribosomes would have to transit across the E1-binding domain for She2p to reach a termination codon at site B. If She2p bound at E1 slows translation, as proposed earlier (Chartrand *et al.* 2002), this could result in inefficient termination at downstream site B. It has been shown that reduced rates of translational elongation cause inefficient termination leading to readthrough (Sandbaken and Culbertson 1988). Since efficient termination is required for NMD (Bonetti *et al.* 1995), sensitivity to NMD might be negatively affected.

The nonsense mutation at site C was insensitive to NMD in all strain backgrounds tested. The insensitivity to NMD could be related to the location of the mutation 356 nucleotides upstream of the normal stop codon (Figure 1). Mutations near the normal stop codon can be insensitive to NMD. For example, the *HIS4* frameshift mutation *his4-713*, which is located 120 nucleotides upstream of the normal stop codon and which generates a premature stop codon immediately after the site of frameshifting, is NMD insensitive (Mathison and Culbertson 1985; Leeds *et al.* 1991). The proposed explanation for insensitivity is that A/U-rich downstream sequence elements required for NMD are not present in between the 3′-proximal premature stop codon and the normal stop codon (Ruiz-Echevarria and Peltz 1996). We identified a potential downstream element, but it might not be functional. In that case, cells might recognize the *ash1-C-ns1* premature stop codon as a normal stop codon.

When nonsense transcripts produced by alleles carrying mutations at sites A or B fail to anchor, they mislocalize as She2-bound transcripts. However, when *ash1-C-ns1* nonsense mRNA fails to anchor, it dissociates from She2p and mislocalizes without being tethered to the actin cytoskeleton. By the time a ribosome reaches site C, it has passed through and presumably displaced She2p binding at three of the four binding domains, leaving the mRNA tethered only through binding in the 3′-UTR. If termination occurs at the normal stop codon, She2p releases an mRNA anchored in the cortex of the bud tip. However, if termination occurs at site C, the mRNA dissociates from She2p without anchoring. The results suggest the existence of a mechanism to ensure that anchored transcripts can be translated full length.

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